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Rediscovery of the Wild-Extinct Species *Nitellopsis obtusa* (Charales) in Lake Kawaguchi, Japan

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The Charales are especially endangered in Japanese inland waters. Of the 74 charalean green algae taxa known from Japan, about half are on the Japanese red list of threatened or endangered species. Although the charalean species *Nitellopsis obtusa* (Desvaux) Groves once grew in four Japanese lakes, field surveys in 1992–1993 suggested that this species was extinct in the wild in Japan. However, during our 2003 field survey, *N. obtusa* was collected from natural populations growing in Lake Kawaguchi. The thalli had giant bract-cells and stars (asexual reproductive organs), which are the diagnostic characters of *N. obtusa*. One-year-old cultured plants developed oogonia. In addition, a phylogeny developed by using sequences from the gene encoding the large subunit of the enzyme rubisco (*rbc*L) demonstrated that the alga collected from Lake Kawaguchi, a laboratory-maintained sample of *N. obtusa* from Lake Nojiri (Japan) and a German *N. obtusa* specimen formed a robust clade within the Charales.

Key words: Charales, *Nitellopsis*, *rbc*L gene, rediscovery, wild-extinct species.

As the biodiversity of freshwater habitats in Japan shrinks because of water pollution following the economic boom of the 1970s, the charalean green algae seem to be especially endangered. Before the 1970s, taxonomic studies recorded 74 taxa in four genera (*Chara*, *Lamprothamnium*, *Nitellopsis*, and *Nitella*) from Japan (Imahori 1954; Kasaki 1964; Imahori and Kasaki 1977). However, field surveys of Japanese lakes in 1993–1994 found that the number of charalean taxa had been dramatically reduced (Nozaki et al. 1995). The Japanese red list now includes five extinct, one wild-extinct, and 24 threatened taxa from the charalean flora in Japan (Environmental Agency of Japan 2000).

Nitellopsis obtusa (Desvaux) Groves is the largest charalean species found in Europe and Asia between 18°N and 65°N (Wood 1965). In Japan, this dioecious species was previously recorded from four lakes: Lake

Ashinoko (Kanagawa Pref.), Lake Kawaguchi (Yamanashi Pref.), Lake Nojiri (Nagano Pref.), and Lake Yamanaka (Yamanashi Pref.) (Kasaki 1964). However, after field surveys in 1992–1993, Nozaki et al. (1994) concluded that *N. obtusa* was extinct in natural habitats in Japan. However cultures of *N. obtusa* previously collected from Lake Nojiri have been maintained as experimental material in the laboratory of Osaka Medical University (Nozaki et al. 1994). Therefore, in Japan *N. obtusa* is listed as an "extinct species in the wild" (Environmental Agency of Japan 2000).

During our 2003 field surveys, living samples of *N. obtusa* were collected from natural populations growing in Lake Kawaguchi. Here we report field data, morphology, and the gene sequence of the large subunit of rubisco (*rbc*L) for the *N. obtusa* obtained from Lake Kawaguchi.

Materials and Methods

Field surveys of charalean species were made at various sites around Lake Kawaguchi (Minamitsuru-gun, Yamanashi Pref, Japan) in June and October 2003 (Fig. 1). Thalli were collected using a handmade anchor (Imahori 1954), which was thrown into the water from the lakeshore or piers and then dragged along the bottom.

Uni-charalean cultures (STAR strain originating from site 12; Fig. 1) were established by inoculating part of a *N. obtusa* thallus into a 900-ml glass jar containing a soil-water-medium for Charales (SWC-1; Sakayama et al. 2004). The cultures were maintained under controlled laboratory conditions at 20 °C with a 14:10-h light-dark cycle and ca. 140 μmol m^{-2} s^{-1} illumination provided by fluorescent lamps. The thalli collected from site 12 (Fig. 1) were also grown in a 12-L tank containing SWC-1 and were maintained at room temperature (15–30°C) with a 14 : 10-h light-dark cycle and ca. 500 μmol m^{-2} s^{-1} fluorescent lamp illumination.

The thalli were observed with a UFX-IIA microscope (Nikon, Tokyo, Japan) and a MS5 stereo microscope (Leica Microsystems, Tokyo, Japan). Taxonomic identifications were based on Kasaki (1956), Wood (1964, 1965), and Imahori and Kasaki (1977).

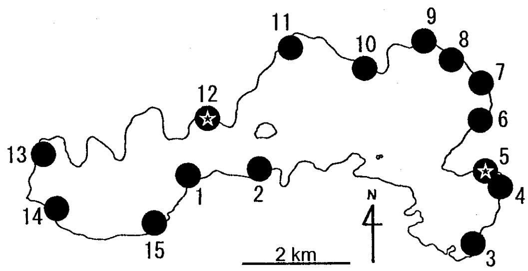

Fig. 1. Map of Lake Kawaguchi, Yamanashi Pref., Japan. Collection sites are numbered 1–15. Collections were made in June 2003 at sites 1–3, 5, 6, 8, 11–13, and 15, and in October 2003 at sites 1, 4, 5, 7, 9, 10, 12, and 14. *Nitellopsis obtusa* was collected at sites 5 and 12.

Table 1. Comparison of the charalean taxa found in Lake Kawaguchi from three surveys in different decades

Kasaki (1964)	Nozaki et al. (1995)	the present study
Chara braunii Gmelin	*C. braunii*	*C. braunii*
C. globularis Thuillier var. *globularis*	*C. globularis* var. *globularis*	*C. globularis* var. *globularis*
		C. corallina Willdenow
Nitella flexilis Agardh var. *flexilis*	*N. flexilis* var. *flexilis*	
N. gracilens Morioka		
		N. hyalina Agardh
Nitellopsis obtusa (Desvaux) Groves		*Nitellopsis obtusa*

Table 2. Species and culture strains used for the *rbc*L gene phylogeny

Spesies	strain designation and collection information	origin of *rbc*L gene sequence data	GenBank accession number
Chara globularis Thuillier	F124C, Germany	McCourt et al. (1999)	AF097163
C. connivens Salzmann ex Braun	X214, Israel	McCourt et al. (1999)	AF097162
C. vulgaris Linnaeus	X152, Denmark	McCourt et al. (1999)	AF097166
C. rusbyana Howe	X066, Argentina	McCourt et al. (1999)	AF097168
Lamprothamnium papulosum (Wallroth) Groves	F137, France	McCourt et al. (1999)	AF097170
Nitellopsis obtusa (Desvaux) Groves	F131, Germany	McCourt et al. (1999)	U27530
	STAR, Lake Kawaguchi, Yamanashi, Japan, Oct. 2003	the present study	AB195319
	NOJIRI, Lake Nojiri, Nagano, Japan, Aug. 1974	the present study	AB195320
Lychnothamnus barbatus (Meyen) Leonhardi	Ger, Germany	McCourt et al. (1999)	AF097172
Nitella flexilis (Linnaeus) Agardh	S010, Japan	Sakayama et al. (2002)	AB076056
N. praelonga Braun	P/CR7, Costa Rica	McCourt et al. (1999)	AF097173
N. pulchella Allen	S011, Japan	Sakayama et al. (2002)	AB076057
N. furcata (Roxburgh ex Bruzelius) Agardh	S037, Japan	Sakayama et al. (2002)	AB076059
N. gracilens Morioka	KINU, Japan	Sakayama et al. (2002)	AB076063
N. translucens (Persoon) Agardh	F108, France	McCourt et al. (1999)	AF097745
Tolypella. nidifica (Müller) Braun	F138, France	McCourt et al. (1999)	U27531
Coleochaete nitellarum Jost		Nishiyama and Kato (1999)	AB013662
C. orbicularis Pringsheim		Manhart (1994)	L13477
Zygnema peliosporum Wittrock		Mccourt and Karol (1995)	U38701

DNA sequencing of the *rbc*L gene of the *N. obtusa* from Lake Kawaguchi (STAR strain) and from Lake Nojiri (NOJIRI strain; Nozaki et al. 1994) was performed as described by Sakayama et al. (2002). For the phylogenetic analysis, a data matrix containing 1194 bp of unambiguously aligned *rbc*L gene sequences from three samples of *N. obtusa*, 13 other charalean species, and three other charophycean species (Table 2) was subjected to unweighted maximum parsimony (MP) analysis using PAUP* 4.0b10 (Swofford 2002). A bootstrap analysis (Felsenstein 1985) was carried out based on 1000 replications of the general heuristic search (full heuristic type with the tree bisection-reconnection branch-swapping algorithm). From the same alignment used in the MP analysis, a distance matrix was calculated by applying the Jukes-Cantor method (Jukes and Cantor 1969) in PAUP* 4.0b10. A phylogenetic tree was then constructed by a neighbor-joining (NJ) algorithm (Saitou and Nei 1987), again using PAUP* 4.0b10. The robustness of the phylogeny was tested by a bootstrap analysis with 1000 replications. In these phylogenetic analyses, *Coleochaete nitellarum* Jost, *C. orbicularis* Pringsheim, and *Zygnema peliosporum* Wittrock were designated as the outgroup because they belong to the Charophyceae sensu Mattox and Stewart (1984), while recent molecular phylogenetic studies give reasonably high bootstrap support for the monophyly of the Characeae within the Charophyceae (McCourt et al. 1999, 2000).

Results

Field collections and cultures — Five charalean taxa including *Nitellopsis obtusa*

were collected for this study (Table 1). The collection sites are shown in Fig 1. In June 2003, a piece of a *N. obtusa* thallus with thickened nodal cells was collected at site 5. After several months, cultures of this sample demonstrated the typical vegetative morphol-

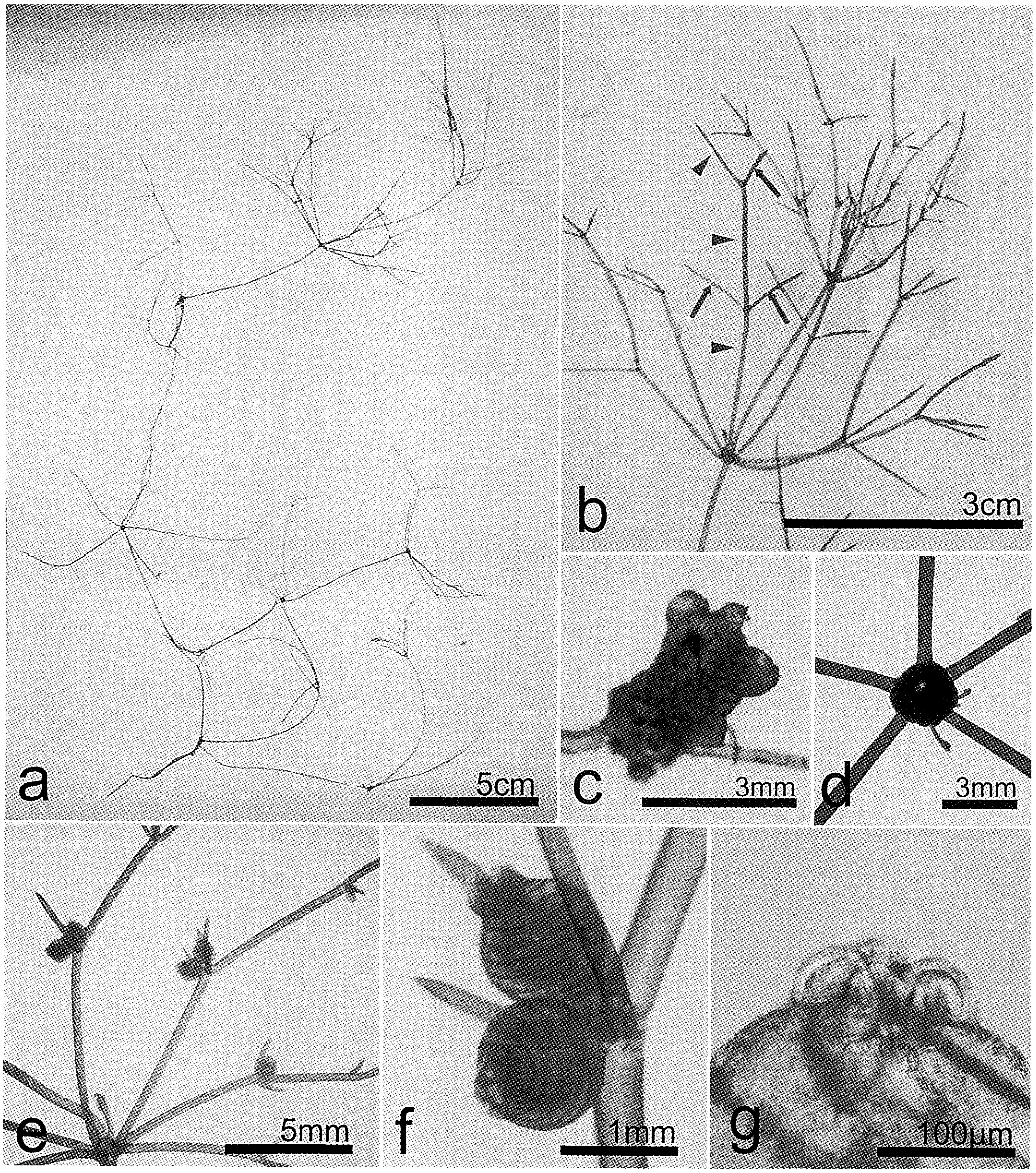

Fig. 2. Morphology of *Nitellopsis obtusa* Groves specimens collected at site 12 in Lake Kawaguchi. a–d. Thalli field-collected in October 2003. a. Habit. b. Apical portion of thallus. Arrows or arrowheads indicate bract-cells or branchlet segments, respectively. c. Stars (asexual reproductive organs). d. Thickened nodal cells. e–g. Oogonia developing in plants cultured in a 12-L tank for about one year. e. Oogonia formed as solitary or geminate at branchlet nodes. f. Oogonia. g. Apical portion of immature oogonium showing coronula composed of five cells arranged in one tier.

ogy of *N. obtusa* (not shown). In October 2003, thalli with the typical vegetative morphology of *N. obtusa* were collected at site 12. No *N. obtusa* was found at site 5 at that time.

Morphology — The morphology of the *N. obtusa* thalli collected at site 12 of Lake Kawaguchi agreed with descriptions given by Kasaki (1956) and Wood (1964, 1965): thalli were 15–40 cm high; axes were up to 1 mm in diameter; and internode cells were 1–1.5 times as long as branchlet cells (Figs. 2a, b). Whorls consisted of five to six branchlets, up to 6 cm long (Figs. 2a, b). Bract-cells were 1–2, similar to the branchlet-segments, and were directed somewhat toward the axis; they measured 1.2–1.7

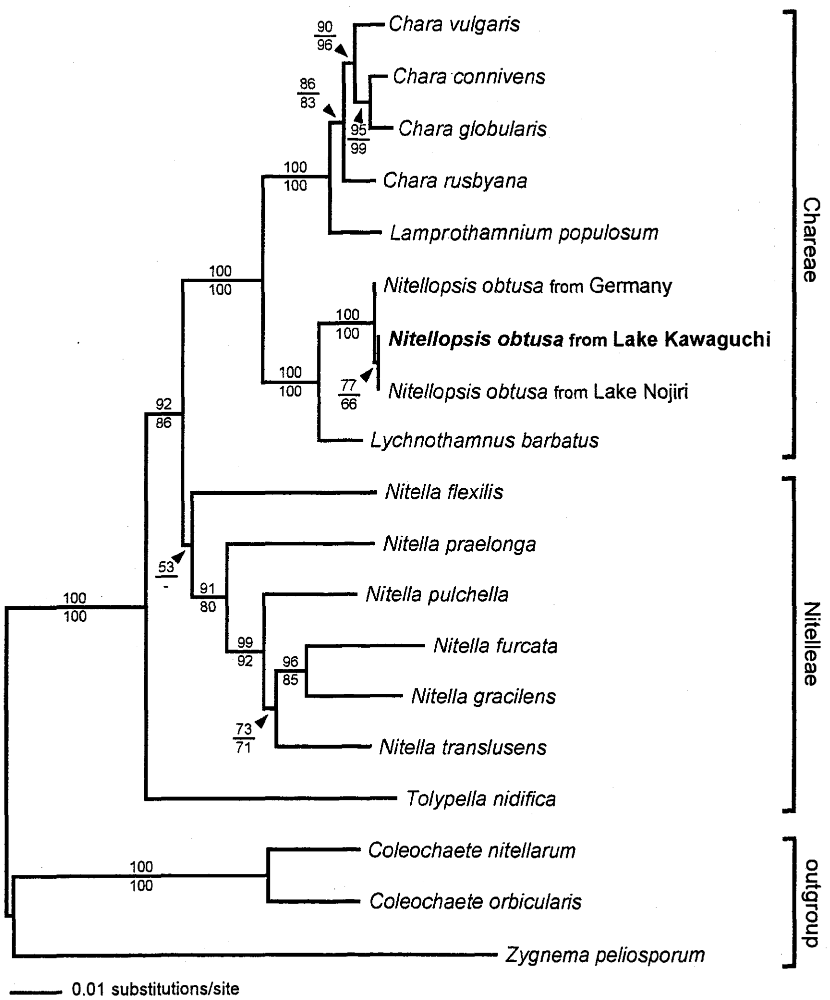

Fig. 3. Distance tree based on aligned nucleotide sequences for 1194 bp of the *rbcL* gene for 16 charalean taxa and three outgroup taxa. The tree was derived using the neighbor-joining method (Saitou and Nei 1987). This method is based on the Jukes-Cantor distance (Jukes and Cantor 1969), which is indicated by the scale bar below the tree. Numbers above or below the branches are bootstrap values (50 % or more) based on 1000 replications of the NJ or MP analyses.

cm long (Fig. 2b). White stars were ca. 3 mm in diameter (Fig. 2c). Nodal cells were thickened and ca. 3 mm in diameter (Fig. 2d).

Oogonia developed on thalli cultured for approximately one year in a 12-L tank. The oogonia, which formed as solitary or geminate at the lowest 1–2 branchlet-nodes, measured 1000–1140 μm long and 870–1050 μm wide (Figs. 2e, f). The coronula were composed of five cells arranged in one tier, measuring 40 μm high and 120 μm wide at the base (Fig. 2g). Antheridia were not observed.

Molecular phylogenetic analyses—The *rbc*L gene sequences of two Japanese plants of *N. obtusa* (from Lake Kawaguchi and Lake Nojiri) were identical. They differed from the sequence of a German *N. obtusa* specimen (McCourt et al. 1996) by only one base pair.

The NJ tree of the *rbc*L gene sequences is shown in Fig. 3. Branches that resolved with bootstrap values of 50 % or more by the NJ and/or MP analyses are shown. The phylogenetic relationships among the six genera of the Characeae resolved in this study were essentially the same as those found by Sakayama et al. (2002). Four genera of the Chareae (*Chara*, *Lamprothamnium*, *Lychnothamnus* and *Nitellopsis*) were resolved as a monophyletic group, whereas two genera of the Nitelleae (*Nitella* and *Tolypella*) represented a paraphyletic group. Within the Chareae, the samples of *N. obtusa* from Lake Kawaguchi, Lake Nojiri, and Germany formed a robust clade with high bootstrap values (100 %) using both the MP and NJ methods (Fig. 3).

Discussion

Our two collections of viable *Nitellopsis obtusa* thalli from Lake Kawaguchi demonstrate that natural populations of the algae are extant. The species was identified based on its morphology (Fig. 2). In addition, a molecular phylogenetic analysis demonstrated that the specimens from Lake Kawaguchi are very closely related to *N. obtusa* specimens originating from Lake Nojiri and Germany (Fig. 3). *Nitellopsis obtusa* habitats in Lake Kawaguchi are, however, much reduced from those reported by Kasaki (1964). He reported that *N. obtusa* was found at several sites circling the perimeter of Lake Kawaguchi (Fig. 1). In contrast, we collected *N. obtusa* at only two of the 15 sites we surveyed around the lake. Furthermore, *N. obtusa* populations may be sporadic or very small, as the alga was not present at the same site in both June and October 2003. Therefore, populations of *N. obtusa* growing in Lake Kawaguchi should be conserved as soon as possible.

The rediscovery of *N. obtusa* in Lake Kawaguchi may be explained by one of two possibilities. First, either oospores (zygotes) or stars of *N. obtusa* embedded in the lake bottom may have germinated. According to Kasaki (1964), *N. obtusa* plants collected from Lake Kawaguchi and Lake Ashinoko were female, whereas those from Lake Nojiri were male. The thalli we collected from Lake Kawaguchi produced only female organs (Figs. 2e–g). *Nitellopsis obtusa* can also reproduce asexually by stars. Therefore, buried stars may have given rise to the vegetative thalli, after the survey of Nozaki et al. (1994). However, the stars do not have a thick cell wall (Wood 1965) and thus may not be viable after a certain time. Second, the rediscovery may result from differences in collection methods. Nozaki et al. (1994) surveyed Lake Kawaguchi only once in 1992, whereas we surveyed the lake on three days in June and October 2003 for the present study.

The charalean taxa found in this survey are different from those collected from Lake Kawaguchi by Kasaki (1964) and Nozaki et al. (1995) (Table 1). Although Kasaki (1964) and Nozaki et al. (1995) collected

Nitella flexilis Agardh var. *flexilis*, we did not find this species. *N. gracilens* Morioka has not been collected from Lake Kawaguchi since Kasaki's survey (1964). In contrast, *Chara corallina* Willdenow and *N. hyalina* Agardh were newly collected in the present study (Table 1). The different taxa represented in the various surveys may indicate recent environmental changes in Lake Kawaguchi and suggests a need for detailed resurveys of current Japanese charalean flora in other lakes (Nozaki et al. 1995).

Since Kasaki (1956) first reported *Nitellopsis obtusa* in Japan, taxonomic identifications of Japanese species have been based solely on morphological attributes (e. g., Kasaki 1964; Nozaki et al. 1994). Our study used *rbc*L gene sequences to demonstrate that *N. obtusa* specimens originating from Lake Kawaguchi, Lake Nojiri, and Germany are very closely related to one another, adding support to the previous morphological identification of Japanese *N. obtusa* collections.

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日本産の車軸藻類はこれまでに4属74分類群が報告されているが，近年の環境汚染による水質の汚染等が原因で，全国で激減しており，レッドデータブックにも多数の絶滅危惧種・絶滅種が記載されている．ホシツリモ（*Nitellopsis obtusa*）は，日本では4湖沼（芦ノ湖，河口湖，野尻湖，山中湖）に生育していたが野生絶滅種とされている．今回，山梨県河口湖において2003年6月，10月に車軸藻類の調査を行ったところ，ホシツリモと思われる藻体が発見された．本藻体はホシツリモに特有な長大な苞と，無性生殖器官である星状体が確認でき，加崎（1956）の記載と一致した．また，培養後1年藻体から雌器（生卵器）が発達した．さらに今回の河口湖産の *rbc*L 遺伝子の塩基配列は，野尻湖産及びドイツ産のものとほぼ一致し，本藻体がホシツリモであることのより確かな確証を得た．現在の河口湖における生育域は非常に減少しており，緊急な保護対策が必要と考えられる．

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